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Procoagulant platelets in haemostasis and thrombosis

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Authors' contributions

S.S., R.K, J.W.M.H and L.N performed research, drafted the paper and contributed to the final paper version.

Disclosures

S.S. is an employee of Synapse Research Institute Maastricht, part of the Stago Diagnostics group. J.W.M.H. is scientific advisor of the same institute.

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Abstract

Procoagulant platelets are characterized by sustained high cytosolic Ca^{2+}, surface exposure of phosphatidylserine (PS) and P-selectin, and a balloon-shaped morphological transformation. These features strongly facilitate the generation of coagulation factor Xa and thrombin on the platelet membrane. Here, we provide a referenced summary of 136 mouse and human genes and proteins, implicated in the formation of procoagulant, PS-exposing platelets. A much smaller set of mouse genes and proteins is reported to be involved in thrombin-induced clot retraction. Based on this literature inventory, we generated an integrated signalling scheme of the multiple membrane receptors and channels, cytosolic, mitochondrial and plasma proteins contributing to platelet procoagulant activity and PS exposure. In the second part of this review, we describe the formation and roles of procoagulant platelets as published for mouse models of haemostasis, thrombosis and thrombo-inflammation. We also overview the evidence for procoagulant platelet formation in human arterial thrombosis, stroke, venous thromboembolism, trauma, COVID-19, and immune-mediated thrombotic disorders. Finally, we define potential therapeutic targets that, once clinically validated, can serve as tailored treatment options to selectively suppress the thrombogenic activity of procoagulant platelets.

Key words: immune thrombocytopenia, haemostasis, phosphatidylserine, platelets, thrombosis, thrombo-inflammation

Platelets interact with the coagulation system in several ways, thus contributing to haemostatic and thrombotic processes, along with inflammatory reactions and malignancies.(1-3) Recent findings emphasize the importance of two of these interactive processes, namely platelet procoagulant activity, hallmarked by surface exposure of phosphatidylserine (PS) and P-selectin, and furthermore thrombin-induced retraction of platelet-fibrin clots. The first process steers PS-dependent thrombin generation, whereas clot retraction serves to consolidate and tighten preformed platelet aggregates.(4-6)

In this paper, we provide a comprehensive overview of the roles of multiple mouse and human genes and proteins that are reported to regulate platelet procoagulant activity, alone or together with clot retraction. In comprehensive signalling schemes, we combine this literature information, aiming to capture the cellular features of procoagulant platelets. Given the partial phenotypic and ontological overlap of procoagulant and apoptotic platelets(5, 7), we also include proteins controlling the apoptotic process in platelets. In the second part of this review, we zoom in on recent knowledge on the roles of procoagulant platelets in haemostatic and thrombotic processes in mouse models, and subsequently in human arterial and thrombotic diseases. Finally, we discuss biomarker evidence and therapeutic opportunities for inhibiting this unique platelet response.

A. Characteristics of platelet procoagulant activity

The capacity to expose PS following maximal activation is a feature of essentially all platelets, provided that the cytosolic Ca^{2+} concentration is raised to supra-micromolar threshold levels, e.g. as achieved with Ca^{2+}-ionophores.(8, 9) In this paper, we adhere to the consensus report, defining agonist-induced procoagulant platelets as flow-cytometric populations expressing both PS and P-selectin.(5) Additional features accompanying PS exposure are: *(i)* platelet swelling to ballooning,(10, 11) *(ii)* proteolytic inactivation of integrin αIIbβ3, and *(iii)* shedding of extracellular vesicles.(12) Figure 1 provides some high-resolution images of key features of these ballooned, procoagulant platelets.

Today, many agonists and receptors are known to contribute to platelet PS exposure. Accordingly, we are not using the term COAT (collagen and thrombin activated) platelets,(13, 14) although it is clear that collagen and thrombin are major agonists of this platelet response. The formation of fibrin-coated platelets and

extracellular vesicle shedding,(15, 16) we consider here as secondary, dampening mechanisms of platelet coagulant activity, though representing important biomarkers. For a description of the characteristics and activation modes of the various platelet types related to procoagulant activity, such as used in the literature, we refer to Table 1. Regarding the platelet proteins implicated in clot retraction,(17) we here confine to a comparison with procoagulant platelets.

B. Genes and mechanisms of platelet procoagulant activity in mouse and human

An extensive search in PubMed ("gene/protein" & platelet & phosphatidylserine/procoagulant or clot retraction) revealed 136 mouse and/or human genes and proteins - out of 3,474 earlier identified genes with a platelet-related phenotype -(18) that have been reported to regulate platelet PS exposure, of which 38 also contribute to clot retraction (lists in Suppl. Figure 1 and Datafile A). This high number of 136, in comparison to earlier reviews,(8, 9, 14) allowed for the first time to construct comprehensive signalling maps of proteins involved in platelet procoagulant activity. For this purpose, we ordered the genes and corresponding proteins along a previous classification scheme of protein location and function (Figure 2A).(19) This revealed that major proportions of the 136 proteins/genes consisted of membrane receptors and channels (class C10), secretory proteins (C17) and signalling proteins (C18) (Figure 2B). Less prominent were the classes of mitochondrial proteins (C11) and metabolic proteins (C12). The few (n=4) mitochondrial proteins implicated in platelet procoagulant activity functioned in particular in mitochondrial Ca^{2+} handling.(18) In apparent contrast, a much larger set of genes encoding for mitochondrial proteins in mouse has a cardiac phenotype, potentially limiting analysis of a platelet phenotype due to mortality and systemic alterations (Mouse Genome Informatics database).

The current literature suggested that only a small proportion of the 136 mouse genes also contributed to clot retraction (black bars in Figure 2B), on the one hand underlining that platelet procoagulant activity is a mechanistically distinct pathway. Typically, actin cytoskeletal proteins (C01), small GTPase regulators (C19) and transcription proteins (C20) contributed almost exclusively to clot retraction (orange bars). On the other hand, it should be stated that the lower number of proteins involved in clot retraction likely is an underestimate due to knowledge gaps, given that many more genes are known to regulate platelet aggregation and thrombus formation

(Figure 2C).

Of the 136 mouse and human genes/proteins contributing to platelet procoagulant activity (Suppl. Figure 1A), 82% are involved in thrombus formation or platelet aggregation, whereas only 48% have a haemostasis (bleeding) phenotype. With the exception of several plasmatic coagulation factors, the vast majority of the genes exhibit high protein expression levels in mouse and human platelets (Suppl. Figure 1A, details in Datafile A).(18) This is particularly the case for the 38 genes implicated in both PS exposure and clot retraction (Suppl. Figure 1B).

In the following section, we discuss how many of the identified genes and proteins contribute to distinct phases of the platelet procoagulant response. Figure 3 depicts a signalling scheme based on the common protein names for human platelets. Figure 4 plots this information for the corresponding mouse gene symbols, aiming to make a direct link to the genes phenotyped in Suppl. Figure 1.

Phase a: co-signalling via ITAM-linked and G protein-coupled receptors (Figure 3a, 4a)

The conventional way of platelet PS exposure is by stimulation of ITAM-linked receptors (ILRs), such as the collagen receptor glycoprotein VI (GP6), CLEC2 or FcγRIIA (FCGR2A), in combination with G protein-coupled receptors (GPCRs), such as the thrombin receptors PAR1/3/4 (F2R, F2rl2, F2RL3).(9) Herein, the role of mouse PAR3 (F2rl2) may be confined to only a regulatory cofactor for PAR4.(20) The combination of receptor stimulations triggers two complementary routes of inositol triphosphate-induced Ca^{2+} release, *i.e.* via phospholipase C (PLC)γ and β isoforms, respectively. Platelet signalling induced by GPVI and co-receptor FcR γ-chain (FCER1G) involves several protein tyrosine kinases (SYK, BTK, SRC), all of which contribute to the prolonged-high Ca^{2+} rise required for PS exposure. Protein tyrosine phosphatases (PTPs) play additional roles herein, with some stimulating and other suppressing the signalling process.(21) A similar duality is seen for the protein kinase C (PKC) isoforms that are also activated via PLCs. As far as understood, PKCα/β (PRKCA/B) isoforms enhance platelet Ca^{2+} rises, whereas PKCδ/θ (PRKCD/Q) are inhibitory.(22) The thrombin-induced GPCR activation via Gqα and PLCβ isoforms induces oscillatory Ca^{2+} rises, which seem to be insufficient for PS exposure.(9)

However, in combination with ILRs, the oscillations change into prolonged high Ca^{2+} responses, ultimately leading to PS exposure. Recent evidence shows that PKC stimulation (likely via isoforms PKCδ/θ effectively suppresses the ORAI1-mediated Ca^{2+} entry process via the phosphorylation of adapter protein BIN2.(23) Given the overall requirement of PKC for platelet integrin αIIbβ3 activation and aggregation, this makes PKC activation a key switch of signalling towards either pro-aggregatory (clot-retracting) platelets or high-Ca^{2+}, PS-exposing platelets.(16, 23)

Phase b: high cytosolic Ca^{2+} rises depending on Ca^{2+} entry (Figure 3b, 4b)

Treatment with Ca^{2+} ionophore (ionomycin or A23187) plus extracellular $CaCl_2$ is a sufficient condition to induce PS exposure and ballooning of essentially all platelets.(4) In a similar way, PS exposure can be achieved by thapsigargin, which suppresses back-pumping of cytosolic Ca^{2+} by the sarco-endoplasmic reticulum Ca^{2+}-ATPases SERCA2b/3 (gene symbols ATP2A2, ATP2A3). The thapsigargin-induced PS exposure requires two support mechanisms, i.e. a primary stimulus causing cytosolic Ca^{2+} release via the inositol triphosphate receptors (ITPR1, ITPR2) and the entry of extracellular $CaCl_2$.(23) Mechanistically, it is known that depletion of the Ca^{2+} stores in the endoplasmic reticulum induces the interaction of STIM1 and BIN2 proteins with the plasma membrane ORAI1 Ca^{2+} channel, thus enhancing the cytosolic Ca^{2+} elevation.(24-26) Due to this coupling of Ca^{2+} store depletion and Ca^{2+} entry, sufficiently high Ca^{2+} rises can be generated to result in PS exposure.(27, 28)

Phase c: negative regulation by Gsα-coupled receptors establishing platelet inhibition (Figure 3c, 4c)

Although direct evidence from knockout mouse models is limited, *in vitro* studies indicate that a main platelet inactivation pathway operates via prostacyclin receptors and cAMP elevation. The (endothelial-produced) prostacyclin thus negatively regulates platelets, and hence suppresses agonist-induced PS exposure.(29) The signalling mechanism involves prostacyclin receptors (PTGIR) acting via Gsα (GNAS), adenylate cyclase (ADCY6), and protein kinase A (PKA) α/β isoforms (PRKACA, PRKACB).(28) The latter are serine/threonine kinases that can phosphorylate a large set of platelet signalling proteins.(30) How cyclic nucleotide-dependent phosphodiesterases and platelet-inhibitory cGMP-dependent protein kinase G

(PKRG1) interfere with procoagulant activity requires further investigation.

Phase d: enforcement by autocrine mediators via GPCRs (Figure 3d, 4d)

Additionally to the roles of GPVI, FcγRIIA and PARs in procoagulant platelet formation, several other GPCRs are able to enhance Ca^{2+} fluxes.(31) These include autocrine-activated receptors for ADP, namely $P2Y_1$ and $P2Y_{12}$ (P2RY1/12), and the thromboxane A_2 receptor TP (TBXA2R). Similarly acting are chemokine receptors such as CXCR4. Signalling here occurs via PLCβ, as described above, or via phosphatidylinositol 3-kinases (PIK3C/D) to steer a network of protein kinases.(32) Downstream kinases that enhance platelet PS exposure include AKT1, MAPK1/7 and STK10. Response enhancement is also reported for the diacylglycerol-triggered Na^+ channel TRPC6.(33) Signalling via chemokines is known to increase under inflammatory conditions, but to which extent this affects PS exposure is still unknown.

The occupation of several other receptors can increase PS exposure, often by incompletely resolved mechanisms. These include the adrenaline receptor (ADRA2A) acting by lowering cAMP levels, and furthermore the receptors CD248, CD36 and FAS (Suppl. Figure 1A). Furthermore, outside-in signalling events mediated by ligand-activated integrin αIIbβ3 and glycoprotein Ib-V-IX, both of which are abundant receptors on mouse and human platelets, contribute to PS exposure. The recognised role of glycoprotein Ib-V-IX in collagen-induced platelet procoagulant activity appeared to rely on von Willebrand factor binding at high shear stress conditions.(34) Altogether, there is a steadily increasing number of ligand-induced receptor mechanisms that contribute to platelet activation(35) and, in extenso, to ILR-GPCR induced procoagulant activity.

Phase e: mitochondrial collapse and ion fluxes inducing PS exposure (Figure 3e, 4e)

After the first observations by Jobe and colleagues,(36) multiple studies have pointed to mitochondrial collapse as a requirement for platelet PS exposure and ballooning. The proposed mechanism is opening of the mitochondrial permeability transition pore (MPTP), such as induced by the protein cyclophilin D (PPIF). The current concept is that upon high threshold levels of cytosolic Ca^{2+}, opening of this pore leads to additional release of mitochondrial Ca^{2+} and other molecules, which results in the triggering of ion and water channels in the plasma membrane. The mitochondrial

calcium uniporter (MCU) protein may play a key role in this process.(37) Further contributing to the high Ca^{2+} signal are Na^{+}-Ca^{2+} (SLC8A1/3) exchangers, operating in reverse mode,(38, 39) whereas aquaporin water channels (AQP1) have been implicated in the swelling of procoagulant platelets.(10)

The actual surface exposure of PS in high-Ca^{2+} platelets is mediated by anoctamin-6 (ANO6, previously TMEM16F), acting as a Ca^{2+}-dependent phospholipid and ion channel.(40) Operation of ANO6 thereby antagonises the action of ATP-dependent phospholipid flippases (ATP11C, ATP8A1) in the plasma membrane, which in resting platelets pump externalized PS inwardly. The swelling and ballooning of procoagulant platelets is further mediated by ion/water fluxes and cytoskeletal destruction by calpain, which was found to have a similar Ca^{2+}-dependency as anoctamin-6.(10, 11) Given that also granule secretion is a Ca^{2+}-dependent process, procoagulant platelets expose secretion markers like P-selectin (SELP).

Phase f: enforcement by extracellular thrombin generation (Figure 3f, 4f)

Thrombin, as a co-agonist for platelet procoagulant activity acting via PAR1 receptors, is itself formed on the surface of PS-exposing platelets.(4, 28) Accordingly, coagulation stimulation occurs by the PS-dependent assembly of the tenase complex (factors VIIIa, IXa, X), and the prothrombinase complex (factors Va, Xa, prothrombin), which jointly enhance the generation of thrombin by several magnitudes.(41) Under static conditions in platelet-rich plasma or whole blood, the thrombin generation process allows >100 nM thrombin to be formed.(42) Also under conditions of flow, thrombin is generated on the surface of PS-exposing platelets, thus contributing to procoagulant responses of adjacent platelets.(32)

Phase g: procoagulant termination by fibrin coat formation and extracellular vesicle shedding (Figure 3g, 4g)

Secondarily to PS exposure, a substantial fraction of the platelets assembles a protein coat, which encapsulates fibrin and several platelet-derived proteins, cross-linked to the membrane by transglutaminases such as factor XIII (F13A).(13, 15) These coated platelets, with a declined procoagulant activity but likely longer-term presence in the circulation have been used as valuable biomarkers for PS-exposing platelets. The same can be said of PS-positive extracellular vesicles (EVs), considered to be shed from ballooned platelets,(10) which essentially fragment the platelet surface and have

relatively short circulation times.(43) In genetically modified mice, the extracellular vesicle formation following procoagulant activity is insufficiently investigated, with only an incidental report published.(44)

Phase h: partial overlap with apoptotic and ageing platelets (Figure 3h, 4h)

As far as known, there is an incomplete separation between agonist-induced and apoptotic PS exposure of platelets.(5) Thus, the proapoptotic BH3-mimetic ABT-737 appeared to induce platelet PS exposure in both Ca^{2+}-dependent and caspase-dependent ways.(7) The apoptotic pathway, known to be triggered upon platelet ageing, is regulated by the anti-apoptotic proteins BCL2 and BCL2L1 equilibrating with the proapoptotic proteins BAX, BAK1 and BNIP3L.(28) Under pro-apoptotic conditions (ABT-737), this pathway leads to a controlled activation of proteases of the caspase family, culminating in proteolytically active caspase-3 (CASP3). The PS scramblase activated by caspases is the transmembrane protein XKR8.(45) Current knowledge is that proapoptotic priming contributes to a gradual increase in procoagulant activity of ageing platelets.

C. Procoagulant platelets in haemostasis models

The Scott syndrome is a rare autosomal bleeding disorder that constitutes a direct human genetic model of selective procoagulant platelet dysfunction. The syndrome is recognized by a reduced prothrombin consumption in serum,(46, 47) and the few known patients are compound heterozygous for mutations in *ANO6* (previously *TMEM16F*), encoding for the Ca^{2+}-activated PS and ion channel anoctamin-6.(7, 48, 49) In platelets from affected individuals, stimulation with either Ca^{2+}-ionophore or strong receptor agonists results in low or absent PS exposure. Additional phenotypic consequences are diminished ballooning and a reduced release of PS-exposing extracellular vesicles.(11, 40, 43) In tissue factor-triggered plasma, Scott platelets showed impaired tenase and prothrombinase activities and, hence, a markedly lower thrombin generation.(41, 49) In at least one patient, the platelet ballooning defect could be explained by lower calpain expression and reduced cytoskeleton cleavages.(11, 50)

The clinical haemorrhagic phenotype of the few Scott patients is mild relative to the severity of the *in vitro* coagulation defect. The patients present with provoked rather than spontaneous epistaxis and bleeding. Typical other features are

menorrhagia, and perioperative or post-partum haemorrhage, but spontaneous joint bleeds or intracranial haemorrhages as in haemophilia are not observed.(27, 46, 47) Mouse studies show a reduced, background-dependent vitality upon *Ano6* deficiency, and functional takeover of analogue protein anoctamin-5 (Ano5) in endothelial cells.(51)

The haemostatic function of agonist-induced platelet PS exposure has been examined in germline and platelet-specific knockout mouse models (Datafile 1). In the following, we focus on two mouse genes, encoding for mitochondrial cyclophilin D (mouse gene *Ppif*) and for anoctamin-6 (*Ano6*). In germline $Ppif^{-/-}$ mice, which lack MPTP formation in all tissues, tail bleeding times are indistinguishable from wild-type controls, indicating that PPIF-mediated procoagulant platelet formation is dispensable for haemostatic plug formation upon vascular injury.(36) Consistent with this, platelet-selective PPIF deletion ($Ppif^{fl/fl}$ $Pf4^{Cre}$) likewise leaves haemostasis intact.(52, 53) The picture for anoctamin-6 is less clear; with some studies using germline and targeted *Ano6* deficiency ($Ano6^{fl/fl}$ $Pf4^{Cre}$ with platelet/megakaryocyte gene defect) reported increased tail bleeding times,(54, 55) whereas other studies using conditional knock-outs did not show increased bleeding.(53, 56) Explanations are genetic background effects,(11) and the non-redundant roles of other anoctamins.(57) Although specific CRISPR/Cas mutations have not yet been made, one can consider the germ-line defects may better phenocopy the consequence of human compound heterozygous mutations, such as present in Scott patients.

Collectively, the no more than mild bleeding phenotype in Scott patients and strains of *Ano6*-deficient mice suggests an overall limited role of platelet procoagulant activity in normal haemostasis. Further clarification will come from other mouse bleeding models, e.g. with traumatic or large microvascular injuries such as liver laceration models. These will need to elucidate the importance of procoagulant platelets in settings of enhanced injury. In this context, we note that platelet procoagulant activity can be a compensatory mechanism to improve haemostasis in patients with haemophilia.(58)

D. Procoagulant platelets in thrombosis models

The phenotypes of murine *Ppif* and *Ano6* deletion models have significantly contributed to our understanding of the role of PS-exposing platelets in experimental thrombosis. In germline $Ppif^{-/-}$ mice, arterial thrombosis was found to be enhanced:

stable occlusion in a photochemical carotid artery injury model was increased, when compared to wildtype mice.(36) This counterintuitive result could be explained by a loss of the negative-feedback mechanism normally exerted by highly activated procoagulant platelets – characterized by integrin αIIbβ3 inactivation, high-level surface PS, and extracellular vesicle shedding – which constrain platelet aggregation and thrombus expansion.(59) Interestingly, a platelet/megakaryocyte-selective PPIF deletion reversed the phenotype: *Ppif*$^{fl/fl}$ *Pf4*^{Cre} mice showed mildly reduced thrombus formation after ferric chloride treatment of the carotid artery or cremaster arterioles.(36, 37, 60)

Genetic deletion of *Ano6* is of interest as it relatively preserves αIIbβ3 activation and blocks only PS exposure.(11) This model thereby differentiates between platelet-mediated coagulation activation and integrin/caspase dependent pathways, which depend on mitochondrial Ca^{2+} release. The integrin inactivation in high-Ca^{2+}, procoagulant wildtype platelets is caused by calpain-dependent cleavage of multiple proteins of the inside-out integrin signalling pathway, implying similar Ca^{2+} dependency of calpains and anoctamin-6 channels.(11, 50) *In vivo* experimental data showed that platelet-selective deletion of anoctamin-6 impaired arterial thrombus formation: half of the *Ano6*$^{fl/fl}$ *Pf4*^{Cre} mice failed to produce stable occlusive thrombi in a ferric chloride carotid model, with the remainder showing recurrent embolisation.(53, 54) Venous thrombosis is traditionally targeted by anticoagulants, but new experimental data also indicate a role of platelets.(61) A recent mouse study proved that platelets can turn procoagulant upon vena cava ligation, and that either *Ppif* or *Ano6* deficiency as well as carbonic anhydrase inhibition (targeting procoagulant platelets) partially protect from venous thrombosis.(6) It is important to note that current antiplatelet agents mainly target platelet aggregation, a process that is less critical for low-shear venous thrombus formation.

E. Procoagulant platelets in thrombo-inflammation models

Models of inflamed pulmonary and mesenterial vessels have also provided evidence for a role of procoagulant platelets in the setting of thrombo-inflammation. Using intravital microscopy and a lactadherin-based PS reporter, it was demonstrated that single platelets, patrolling at endothelial breaches and encountering subendothelial collagen, undergo the procoagulant transformation in a cyclophilin D- and anoctamin-6-dependent manner.(53) Such platelets generate a local thrombin burst that seals the

endothelial defect. Furthermore, platelet-selective genetic deletion of either *Ppif* or *Ano6* aggravated pulmonary haemorrhages in mice which were challenged with lipopolysaccharide, without affecting neutrophil recruitment.(6) In ageing, apoptosis-prone platelets, the inflammatory functions appeared to be enhanced.(62)

Regarding ischaemia-reperfusion models, $Ppif^{fl/fl}$ $Pf4^{Cre}$ mice showed reduced infarct volumes, enhanced cerebral blood flow, and improved neurological and motor outcomes upon transient middle cerebral artery occlusion (tMCAO).(52) These benefits were accompanied by reduced platelet-neutrophil aggregate formation, thus placing PPIF-dependent platelet procoagulant activity upstream of the platelet-neutrophil crosstalk.(63) On the other hand, $Ano6^{fl/fl}$ $Pf4^{Cre}$ mice did not show detectable effects on infarct volume, neurological score and motor function in the tMCAO stroke model.(54) This anomaly may be explained by extensive wire-induced vascular injury, massively generating PS-exposing vascular cell membranes, as well as by functional redundancy of other anoctamin isoforms.

Altogether, we foresee that a targeted inhibition of platelet PPIF can limit the ischaemic brain injury without compromising haemostasis. However, further experiments are required to uncover the underlying factors that affect this outcome.

F. Procoagulant platelets in human thrombotic diseases

Clinical evidence for a role of procoagulant platelets in human thrombotic disorders comes from several studies employing different sets of assays, where either PS-exposing platelets or coated platelets are detected, or the procoagulant tendency is measured in patient platelet-rich plasma (Table 2). As described in part B, we consider fibrin-coated platelets with reduced procoagulant activity,(15) along with platelet-derived PS-exposing extracellular vesicles as biomarkers of platelet procoagulant activity.

Arterial thrombosis and ischemic stroke

From the available data regarding human arterial thrombosis and stroke, two take-aways emerge. Numbers of circulating coated platelets appeared to be associated with acute thrombotic and predicted recurrent events (Table 2). Thus, after stroke or transient ischemic attacks, levels of coated platelets appeared to be increased.(64, 65) In patients with asymptomatic internal carotid artery stenosis, increases in coated platelets correlated with the risk on subsequent ischemic stroke or transient ischemic

attack.(66, 67) Also in patients presenting with lacunar or non-lacunar ischaemic strokes, increased coated platelet levels associated with recurrent ischaemic events and a worsened neurological recovery.(67) Rises in coated platelets were also observed in blood from patients with coronary artery disease,(68) but not in patients with cerebral haemorrhages.(66, 69) These findings are compatible with a biomarker indicator function for secondary adverse thrombotic events. Taken together, the few available studies support the idea of targeting procoagulant platelets in pathological arterial thrombosis.

Venous thromboembolism and trauma

A possible role of procoagulant platelets in deep venous thrombosis and pulmonary embolism has long been underestimated, partly because conventional antiplatelet agents targeting thromboxane A_2 and ADP receptor signalling hardly provide protection against venous thrombus formation, and partly because the haemodynamic environment of the venous circulation offers limited extracellular matrix contact to induce procoagulant platelet activation. On the other hand, thrombin is a main player in venous thrombotic clotting and hence acts as platelet agonist. A recent translational study though pointed to important roles of platelets in venous thrombosis (Table 2). Using flow cytometry with triple-positive gating for CD41, PS, and P-selectin, higher circulating procoagulant platelet levels were detected in patients with confirmed deep venous thrombosis or pulmonary embolism, compared to individuals who had equivalent clinical suspicion and D-dimer elevation but negative imaging.(6) In addition, histological analysis of retrieved human pulmonary emboli demonstrated PS-exposing platelets within the thrombus, consistent with an active procoagulant activity in the growing venous clot.

As described above, platelet ballooning is a hallmark of procoagulant activation, driven by ion and water influxes, following Ca^{2+}-mediated activation of calpain cytoskeletal cleavage. Recently, a new trigger mechanism of platelet activation was proposed to contribute to the ballooning.(70) Following major trauma, extracellular histone H4 proteins, as damage-associated molecular patterns released after traumatic cellular injury, were shown to accumulate in peripheral blood and to correlate with incident procoagulant activation. Mechanistically, it was shown that the histones induced sustained cytosolic Ca^{2+} elevation and ensuing ballooning. The mechanism although incompletely resolved relied on neutrophil activation, S100A8/A9 release and

platelet GPIb-V-IX effects.(71) Anti-histone antibodies (via FCRG2A) and histone-enhanced thrombin generation could play a role herein as well.(72)

COVID-19, acquired thrombocytopenias as immune-mediated thrombotic disorders

Immune complex-mediated procoagulant platelet formation is emerging as a novel mechanism contributing to clinically important prothrombotic and thrombocytopenic disorders (Table 2). In patients with severe COVID-19, circulating procoagulant platelets were found to be elevated relative to healthy controls and mildly ill patients, in a manner correlating with plasma D-dimer levels.(73) Mechanistically, the patient IgG-type immunoglobulins engage the platelet ILR FcγRIIA (FCGR2A) to activate signalling, as depicted in Figure 3, and resulting in procoagulant transformation.(74) In line with this, pharmacological or antibody-mediated blockade of immune receptors resulted in suppressed thrombus formation along with procoagulant activity, both *in vitro* and in suitable mouse models.(73) Elevated in severe COVID-19 is also the plasma level of neutrophil-derived calprotectin (S100A8/9), which can engage platelet GPIb-V-IX receptors and prime for procoagulant activity.(71)

In heparin-induced thrombocytopenia (HIT), immune complexes composed of heparin and platelet factor 4 (PF4) also engage platelet FcγRIIA to provoke a similar mechanism of Ca^{2+}/calpain-dependent procoagulant activation.(75) Given the high thrombotic risk of HIT, relative to other thrombocytopenic conditions,(76) the increased PS exposure was proposed as an indicator of the disease severity.(77) In line with this, pharmacological or genetic interference of PS exposure attenuated the HIT immune complex-driven thrombus formation *in vitro*. An analogous mechanism may operate in vaccine-induced immune thrombocytopenia and thrombosis (VITT), in which high-titre antibodies targeting PF4, here independently of heparin, bind FcγRIIA and then induce procoagulant platelet formation.(78) In the latter condition, also neutrophil activation and neutrophil extracellular traps may contribute to the thrombotic process.(79) These findings converge to the conclusion that PS-exposing platelets are to be considered as distinct drivers of a broad variety of thrombogenic disorders.

Procoagulant platelets as clinical biomarkers of thrombotic diseases

Although systemically detectable across thrombotic diseases,(6, 68, 78) levels of procoagulant platelets in the peripheral blood are generally low. This low abundance

underestimates the *in vivo* generation for several reasons. First, procoagulant platelets are mostly formed locally and retained at the site of vascular injury or within a growing thrombus. Second, once circulating the PS-exposing platelet membranes are recognised by peripheral scavenging receptors, triggering removal from the blood, and resulting in a short intravascular lifespan. Third, PS-exposing platelets are fragile and prone to pre-analytical loss. The peripheral count is therefore to be considered a conservative snapshot of a spatially and temporally localised process.

On the other hand, agonist-induced PS exposure as measured *in vitro* reports on the propensity of a person's platelets to become procoagulant. As reviewed elsewhere,(3, 16) this tendency can be determined by genetic factors (Scott syndrome) and the disease state. The *in vitro* measurement is informative for understanding diseases with a thrombosis risk, but does not by itself demonstrate ongoing *in vivo* procoagulant activity. Accordingly, the (patho)physiological relevance of procoagulant platelets should be judged on three axes: (i) analytical rigor with attention to pre-analytical variables; (ii) biological plausibility considering localisation at the vessel wall or thrombus; and (iii) clinical plausibility and relevance. Strongest inference will come from combining the information pieces and anchoring these to clinical endpoints. A limitation in the field though is a lack of consistent nomenclature with interchangeable use of PS-exposing, COAT and coated platelets.

G. Perspectives and clinical intervention

Commonly antiplatelet agents used in the clinic, including $P2Y_{12}$ inhibitors targeting autocrine ADP (clopidogrel, ticagrelor) and aspirin targeting thromboxane A_2 synthesis or blocking activated αIIbβ3 (tirofiban), mainly reduce platelet aggregation. While exhibiting high antithrombotic efficacy, these agents also come with a bleeding risk. We hypothesize that the specific targeting of platelet procoagulant activity can provide an alternative approach of antithrombotic treatment with possibly lower risks of bleeding. We though note that this hypothesis requires further investigations. Given the data presented above, suitable therapeutic targets to modulate platelet procoagulant activation are the ORAI1 Ca^{2+} channel, mitochondrial-directed cyclophilin D and furthermore aquaporins. From a receptor point of view, especially the ILRs GPVI and FcγRIIA and the downstream protein tyrosine kinases will be appropriate targets of anti-procoagulant drugs.

Cyclosporin A is known as a potent inhibitor of cyclophilin D-dependent

procoagulant activity, and is clinically used as an immunosuppressant,(80) making its broad and prolonged use as antithrombotic agent impractical. Gain-of-function mutations in ORAI1 or its regulator STIM1 are observed in the Stormorken syndrome – characteristically showing thrombocytopenia and myopathies –, where platelets are increased in procoagulant activity. Some ORAI1-directed inhibitors are tested in preclinical studies to prevent pancreatitis(81) or specific cancers(82), suggesting the possibility of repurposing for antithrombotic indications. Regarding aquaporins, therapeutic efficacy is shown for the drug acetazolamide in rheumatoid arthritis,(83) and decompensated heart failure,(84) while it is also described in case reports of patients with thrombotic complications.(85)

Regarding GPVI-directed drugs, potential benefit in arterial thrombotic complications and ischaemic stroke has been proposed (86, 87) with a possible extension to venous thrombosis.(88) The targets of multiple tyrosine kinase inhibitors (TKIs) that are broadly used in haematological malignancies (mantle cell lymphoma and chronic lymphoid leukaemia), are highly expressed in platelets.(89) For instance, the SYK inhibitor fostamatinib and the BTK inhibitor ibrutinib are widely prescribed. Although first-line BTK inhibitors appeared to have bleeding side effects, more selective drugs like remibrutinib are haemostasis silent.(90) Therefore, at appropriate dosing, such compounds may also be useful to suppress PS-exposing platelets.

In summary, procoagulant platelet activity appears as a unique, highly dynamic cellular state that, while conserved in mouse and human and observed in various disease entities, has not yet been targeted clinically. While the concept of selective procoagulant targeting holds therapeutic promise regarding antithrombotic efficacy and possibly reduced haemorrhage risk compared to currently used antiplatelet therapy, several major points will need to be addressed by the field. These include: *(i)* standardised diagnostic profiling of both the circulating procoagulant platelet population as well as procoagulant potential, *(ii)* generation of platelet-selective inhibitors that, unlike currently used compounds like cyclosporin A, show low off-target effects when systemically applied; and *(iii)* validating therapeutic efficacy of such compounds in clinically relevant large animal models. Once these obstacles are overcome, future antithrombotic strategies - especially for patients at high bleeding risk - may include personalised, procoagulant platelet-targeting therapy regimen.

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Table 1. Description of platelet types used in this review. Note that these types can overlap, and can be differently named in some papers.

Type, specification	Description
Apoptotic platelet	ABT-737 activated and PS exposing
Ageing platelet	prolonged in circulation, apoptotic tendency
COAT platelet	collagen (peptide) & thrombin-activated, or named so in paper
Coated platelet	transglutaminase-dependent protein coated (FXIIIa, FVa or serotonin probes), or named so in paper
Procoagulant or PS-exposing platelet	platelet agonist-induced PS and P-selectin exposing, ballooned

Table 2. Human thrombotic diseases with reported evidence for elevated procoagulant platelets or extracellular vesicles. Full data and references are given in Datafile B. *Abbreviations:* CP, coated platelets; EV, extracellular vesicles; PM, procoagulant monocytes; PP procoagulant platelets; ↑, increased..

Disease entity	Measured entity	Ref.
Acute respiratory distress syndrome	primed platelets ↑	(91)
Antiphospholipid syndrome	PP, PM ↑	(92)
Deep venous thrombosis	PP, also in thrombi ↑	(6)
Heparin-induced thrombocytopenia	CP, PP formation ↑	(93, 94)
Ischemic stroke	CP ↑	(64, 66)
Lacunar/non-lacunar brain infarction	CP ↑	(67, 95)
Myocardial infarction	CP ↑	(96)
Pulmonary embolism	PP, also in thrombi ↑	(6, 66)
Sepsis-induced coagulopathy	EV (PP) ↑	(97, 98)
Severe COVID-19	PP formation ↑	(73, 99)
Thrombotic thrombocytopenic purpura	EV (PP) ↑	(100)
Transient ischemic attack	CP ↑	(64)
Trauma-induced coagulopathy	PP formation ↑	(70)
Vaccine-induced thrombotic thrombocytopenia	PP formation ↑	(78)

Figure Legends

Figure 1. Identification of procoagulant platelets. (A) Single adherent, retracting, migrating, and procoagulant (PS-exposing) platelets on a fibrin(ogen) coating. Scale bar 3 μm. **(B)** Classification of procoagulant platelets according to SSC-ISTH criteria by flow cytometry: P-selectin (CD62P) positive and annexin A5 positive (marking surface exposure of PS). (**C**) Additional features of procoagulant platelets, i.e. balloon-like morphology, staining positively for annexin A5 and thrombin, produced by the prothrombinase complex. Scale bar 10 μm. **(D)** Procoagulant, ballooned platelets formed on collagen/fibrinogen by combined signalling via GPVI and integrin αIIbβ3. Scale bar 5 μm. Adapted from Ref.(1)

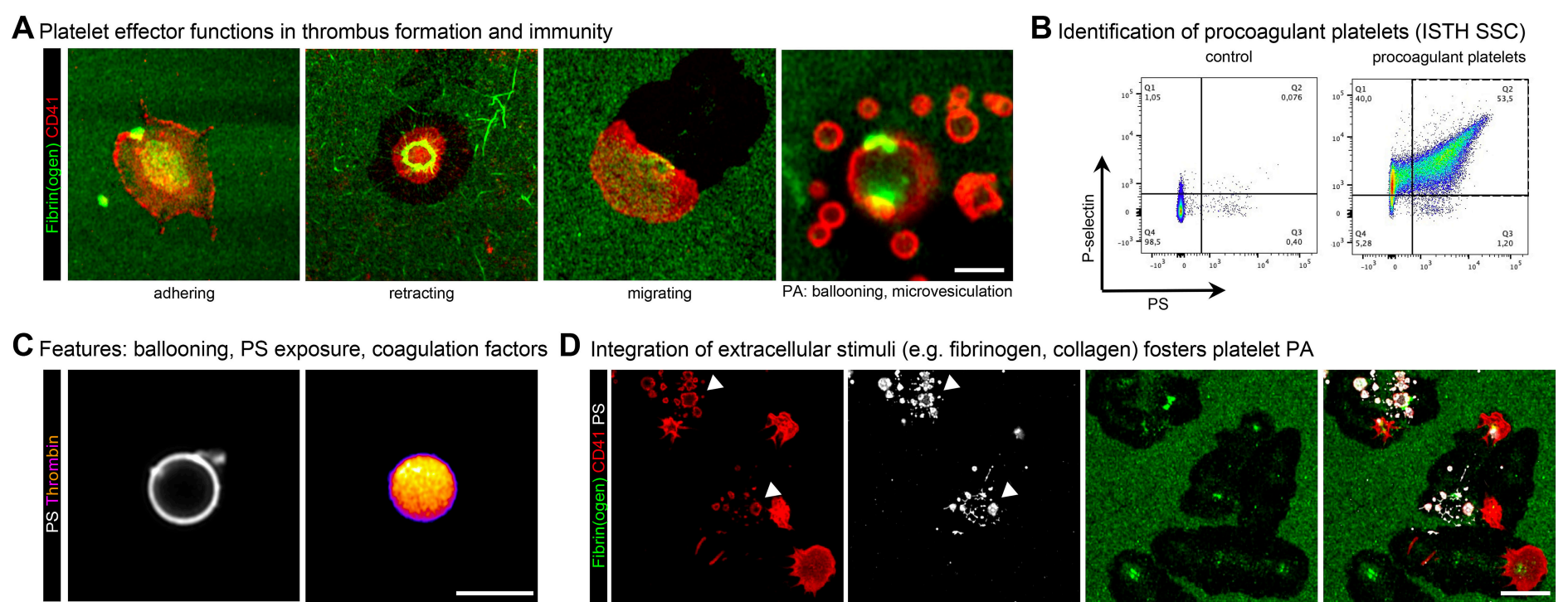
Figure 2. Mouse genes and proteins contributing to platelet PS exposure or clot retraction. (**A**). Classification of genes/proteins per preferred intracellular localisation and function (classes C01-C21) was as described before, along with phenotype descriptions of mouse genes/proteins involved in platelet aggregation and thrombus formation.(18) For the present purpose, the earlier 3474 identified mouse and human genes with a platelet-linked phenotype were subjected to a search in PubMed ("gene/protein" & platelet & phosphatidylserine/procoagulant or clot retraction, +secondary, April 2026). Gene-related phenotype links were rates according to the original paper (see Datafile A). Eligible criteria, genetic models, pharmacological evidence, and platelet expression levels are all described in that paper, along with the matching of orthologous human and mouse genes. (**B**) Numbers of identified mouse genes arranged per class C01-C21 contributing to platelet PS exposure, clot retraction or both. Black bars represent numbers of mouse genes with platelet PS exposure as well as clot retraction phenotypes. (**C**) Similar data as in panel B, but also indicating (white bars) numbers of mouse genes contributing to platelet aggregation or thrombus formation.(18) Detailed phenotypic information on the listed genes together with protein abundance in platelets are provided in Suppl. Figure 1 and Datafile A.

Figure 3. Signalling mechanisms of human platelets contributing to procoagulant activity. Participating proteins are obtained from the list provided in Suppl. Figure 1 and Datafile A. **a**. Default agonist-induced PS exposure achieved by

prolonged high cytosolic Ca^{2+} rises triggered by the combination of Gαq protein-coupled receptors (GPCRs) for thrombin (PAR1/4) and ITAM-linked receptors (ILRs) for collagen (GPVI) or immunoglobulins (FcRγIIA). The ILRs operate via a protein tyrosine kinase cascade (SYK, BTK, SRC). Downstream mediators are phospholipase Cβ and γ isoforms, generating inositol trisphosphate (IP_3) and diacylglycerol (DAG), the latter of which acts via platelet-stimulating (αβ) and -inhibiting (δθ) isoforms of protein kinase C (PKC). **b.** Release of reticular Ca^{2+} in the cytosol via IP_3 receptors, coupled to store-regulated Ca^{2+} entry involving the interaction of STIM1 and BIN2 with ORAI1 Ca^{2+} channels in the plasma membrane. **c**. Negative regulation of platelet activation and PS exposure, which operates via the Gαs GPCR IP for prostacyclin, activating adenylate cyclase and the cAMP-dependent protein kinase A (PKA). **d**. Enforcement of platelet activation and PS exposure through GPCRs and ion channels. These include $P2Y_1$ and $P2Y_{12}$ receptors for autocrine ADP, TP receptors for thromboxane A_2, and CXCR receptors for chemokines. Signalling occurs via Gαq and Gαi, and further downstream via PKC and protein kinase networks of AKT and MAPK isoforms. **e**. PKC-Ca^{2+}-dependent CD62P (SELP) expression, followed by cyclophilin (CypD) and Ca^{2+}-dependent opening of mitochondrial permeability transition pores (MPTP), establishing irreversible rises in cytosolic Ca^{2+}, required to activate the PS and ion transporter anoctamin-6 (ANO6), Na^+-Ca^{2+} exchangers (NCX), and water channels (AQP1). The ANO6-induced phospholipid scrambling antagonises the inward pumping of PS, mediated by ATP-dependent flippases. **f.** Assembly and activation of factor Xa (tenase complex of factors VIIIa, IXa and X) and thrombin (prothrombinase complex of factors Xa, Va and II) at the outer surface of PS-exposing platelets. The resulting thrombin generation enhances the PS exposure of adjacent platelets. **g**. Downregulation of procoagulant activity by transglutaminase (factor XIIIa) dependent formation of a fibrin coat. Calpain-induced destruction of the actin cytoskeleton supports the shedding of extracellular vesicles. Not depicted is calpain-mediated inactivation of integrin αIIbβ3. **h**. Partially overlapping phase of apoptosis-induced PS exposure in ageing platelets via the phospholipid scramblase XKR8. Along with Ca^{2+}, cytochrome C isoforms leaked from mitochondria with proapoptotic and antiapoptotic proteins mediate activation of the central apoptotic protease, caspase 3. Cytoskeletal destruction again leads to extracellular vesicle shedding. *Arrow information:* solid black, ion and PS fluxes; dashed green, negative feedback

mechanisms. Generated with BioRender.com

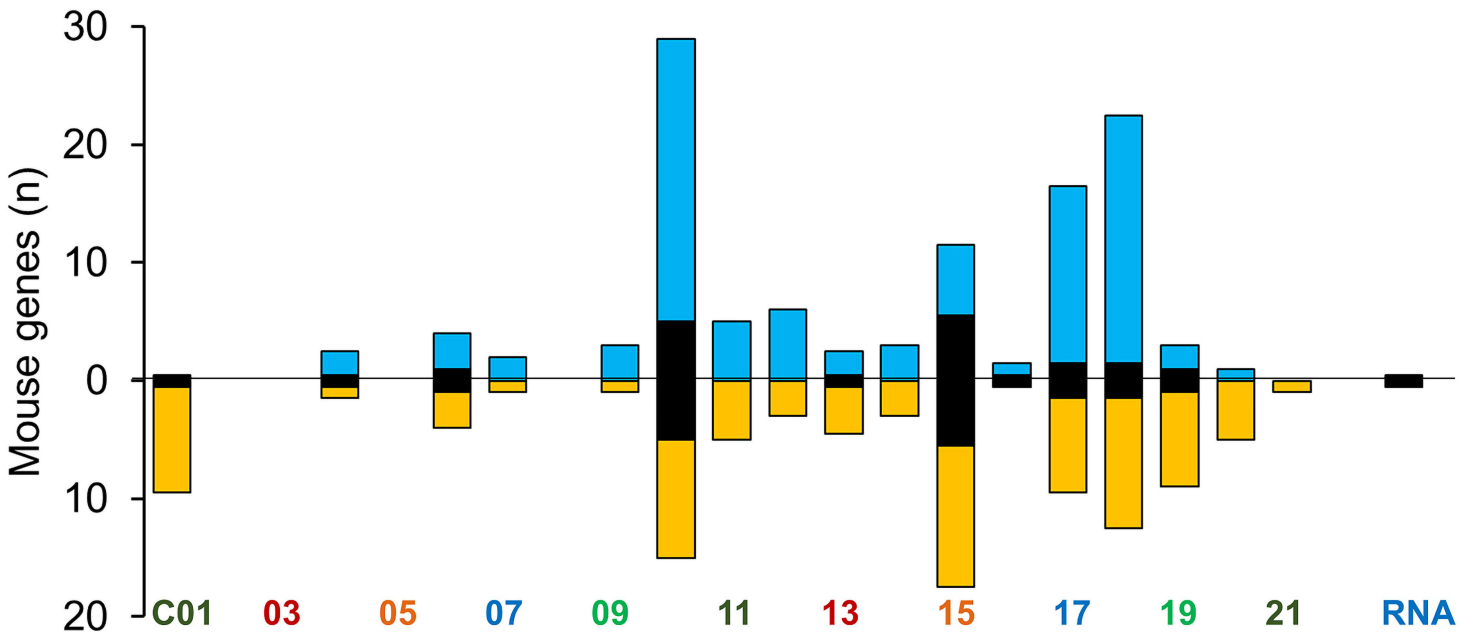
Figure 4. Signalling genes and proteins in mouse contributing to platelet procoagulant activity. Cartoon arranged as in human-based Figure 3, now referring to corresponding mouse genes with a reported phenotype for platelet PS exposure. *Arrow information:* solid black, ion and PS fluxes; dashed green, negative feedback mechanisms. For the phenotype list of mouse and human genes/proteins, see Suppl. Figure 1 and Datafile A. Generated with BioRender.com


A Platelet effector functions in thrombus formation and immunity
Fibrin(ogen) CD41
adhering
retracting
migrating
PA: ballooning, microvesiculation
B Identification of procoagulant platelets (ISTH SSC)
control
procoagulant platelets
P-selectin
PS
Q1 1,05
Q2 0,076
Q4 98,5
Q3 0,40
Q1 40,0
Q2 53,5
Q4 5,28
Q3 1,20
C Features: ballooning, PS exposure, coagulation factors
PS Thrombin
D Integration of extracellular stimuli (e.g. fibrinogen, collagen) fosters platelet PA
Fibrin(ogen) CD41 PS

A

C01	Cytoskeleton actin-myosin	C12	Other metabolism
C02	Cytoskeleton intermediate	C13	Other nuclear proteins
C03	Cytoskeleton microtubule	C14	Proteasome proteins
C04	Cytoskeleton receptor-linked	C15	Protein kinases & phosphatases
C05	Endosome proteins	C16	Protein processing
C06	ER & Golgi proteins	C17	Secretory proteins
C07	Glucose metabolism	C18	Signaling & adaptor proteins
C08	Lysosome & peroxisome proteins	C19	Small GTPases & regulators
C09	Membrane & protein trafficking	C20	Transcription proteins
C10	Membrane receptors & channels	C21	Translation proteins cytosol
C11	Mitochondrial proteins	RNA	RNA genes

B



C

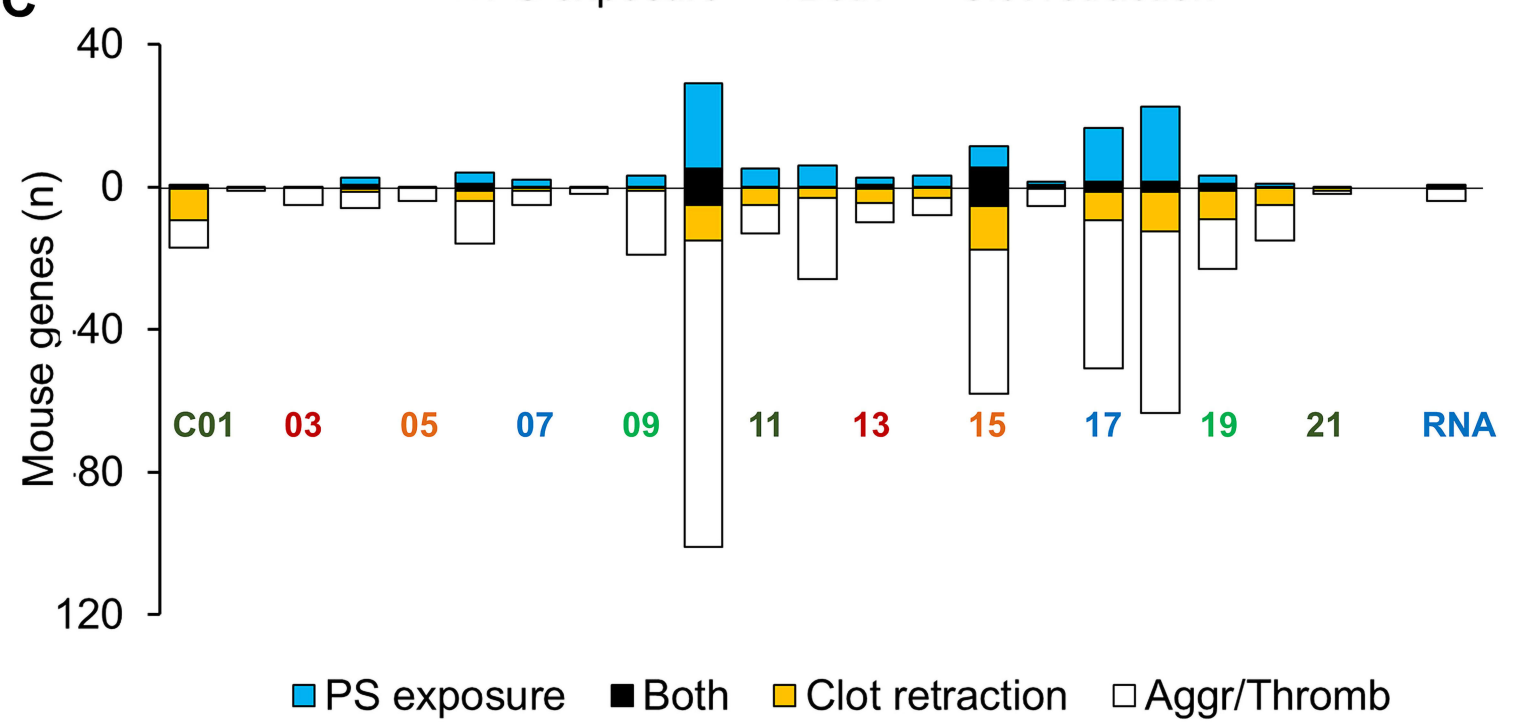


Figure 3

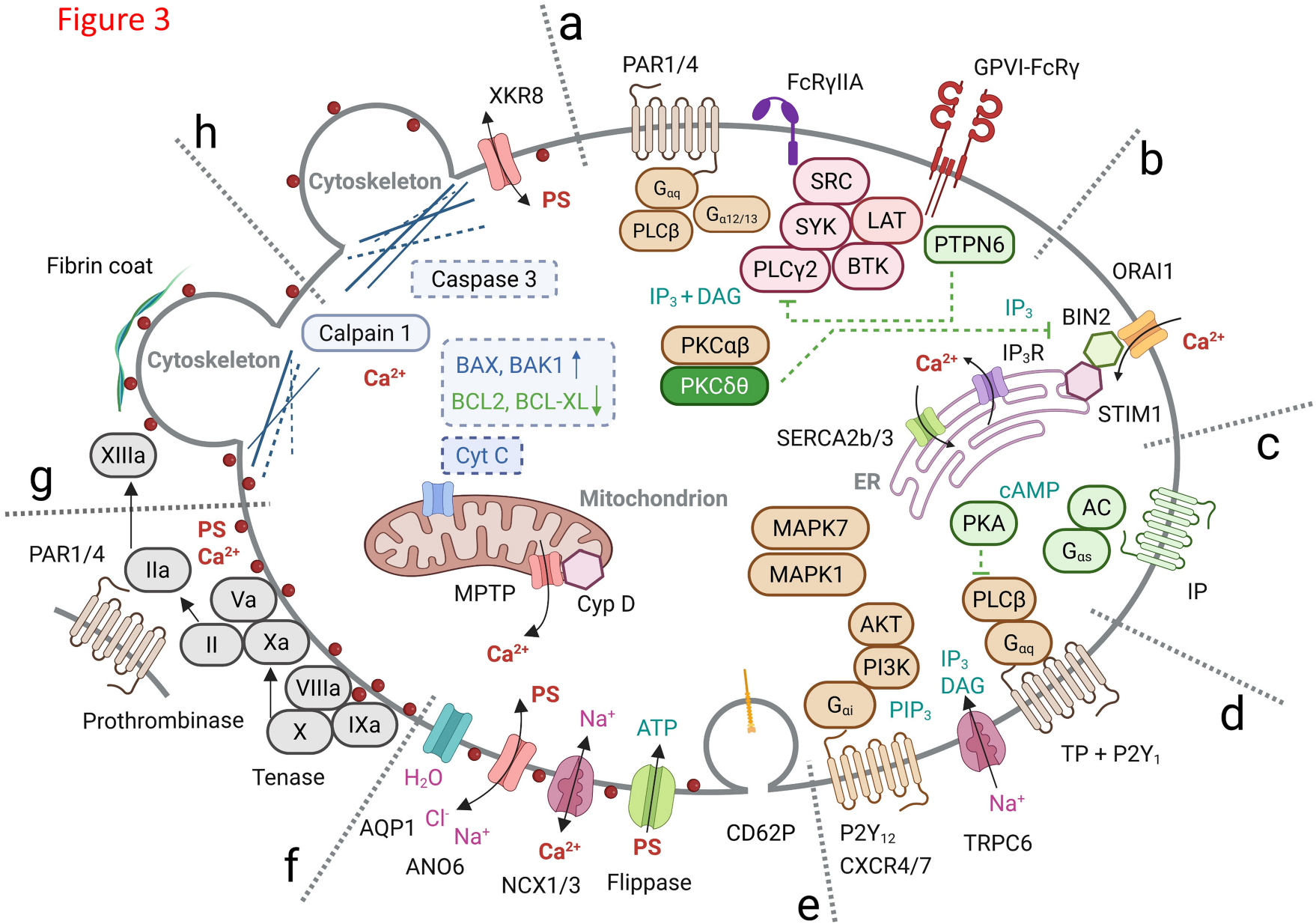
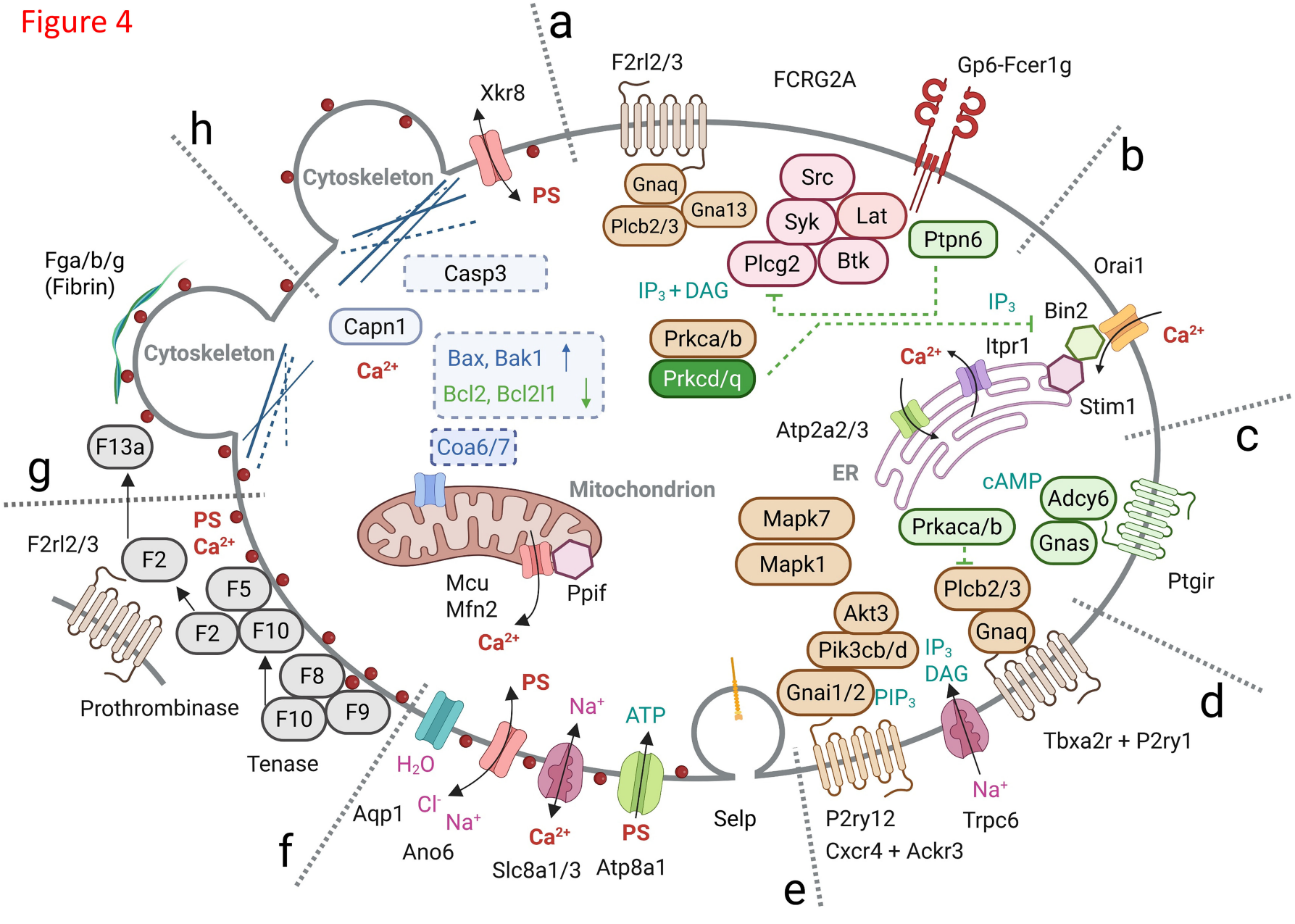

a
b
c
d
e
f
g
h
PAR1/4
FcRγIIA
GPVI-FcRγ
XKR8
PS
Cytoskeleton
Gαq
Gα12/13
PLCβ
SRC
SYK
LAT
PLCγ2
BTK
PTPN6
ORAI1
BIN2
Ca2+
IP3R
STIM1
SERCA2b/3
ER
IP3 + DAG
IP3
PKCαβ
PKCδθ
Caspase 3
Calpain 1
Ca2+
BAX, BAK1 ↑
BCL2, BCL-XL↓
Cyt C
Mitochondrion
MPTP
Cyp D
MAPK7
MAPK1
cAMP
PKA
AC
Gαs
IP
PLCβ
Gαq
AKT
PI3K
Gαi
PIP3
IP3
DAG
TP + P2Y1
P2Y12
CXCR4/7
TRPC6
Na+
CD62P
ATP
PS
Flippase
Ca2+
NCX1/3
Cl-
Na+
AQP1
ANO6
H2O
Tenase
Prothrombinase
XIIIa
Fibrin coat
PAR1/4
IIa
Va
II
Xa
VIIIa
X
IXa
PS
Ca2+

Figure 4


a
F2rl2/3
FCRG2A
Gp6-Fcer1g
Xkr8
PS
Gnaq
Gna13
Plcb2/3
Src
Syk
Lat
Plcg2
Btk
Ptpn6
b
Orai1
IP3 + DAG
IP3
Bin2
Ca2+
Prkca/b
Prkcd/q
Itpr1
Ca2+
Stim1
Atp2a2/3
ER
c
cAMP
Adcy6
Gnas
Prkaca/b
Ptgir
Mapk7
Mapk1
Plcb2/3
Gnaq
Akt3
Pik3cb/d
IP3
DAG
Gnai1/2
PIP3
Tbxa2r + P2ry1
d
P2ry12
Cxcr4 + Ackr3
Na+
Trpc6
e
Selp
PS
Ca2+
Atp8a1
Na+
ATP
H2O
Cl-
Na+
Aqp1
Ano6
Slc8a1/3
f
Prothrombinase
Tenase
F2
F5
F2
F10
F8
F10
F9
PS
Ca2+
F2rl2/3
F2
F13a
g
Fga/b/g
(Fibrin)
Cytoskeleton
h
Cytoskeleton
Casp3
Capn1
Ca2+
Bax, Bak1
Bcl2, Bcl2l1
Coa6/7
Mitochondrion
Mcu
Mfn2
Ppif
Ca2+

Online supplement

Procoagulant platelets in haemostasis and thrombosis

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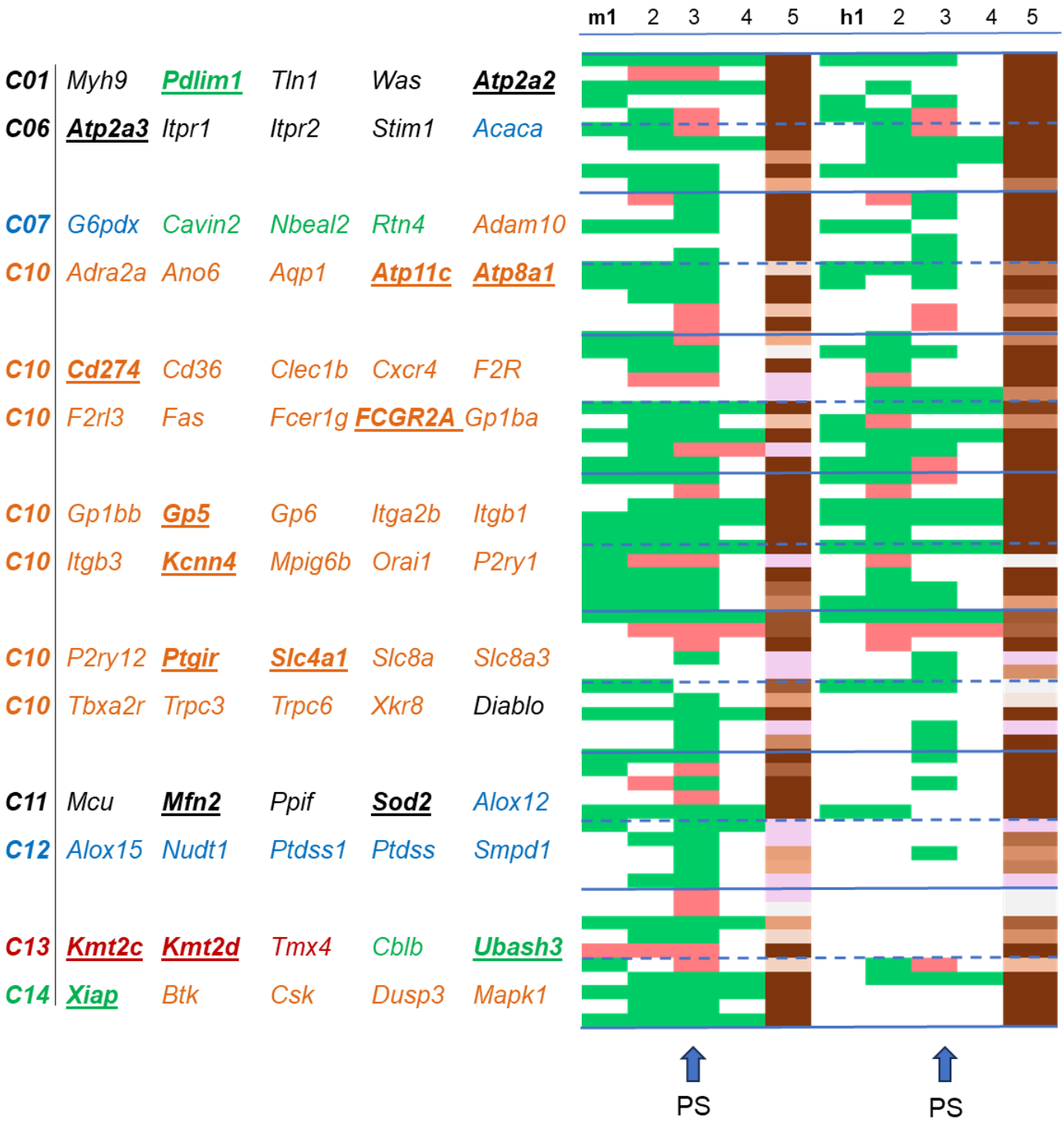
Supplemental figure

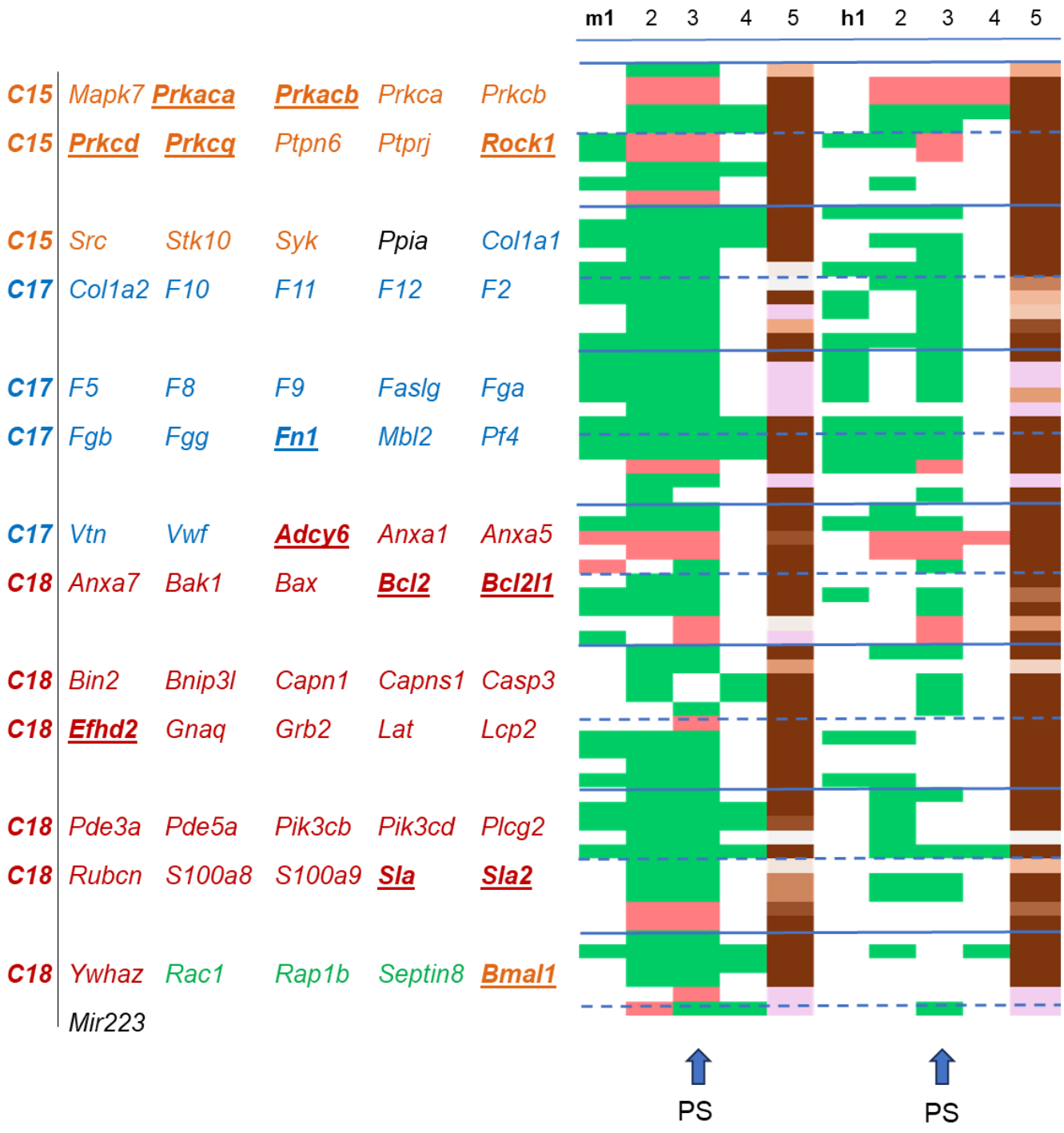
A *Altered platelet PS exposure*



m1: mouse hemostasis low high
m2: mouse thrombosis & platelet aggregation
m3: mouse platelet PS exposure
m4: mouse clot retraction
m5: mouse platelet protein copies

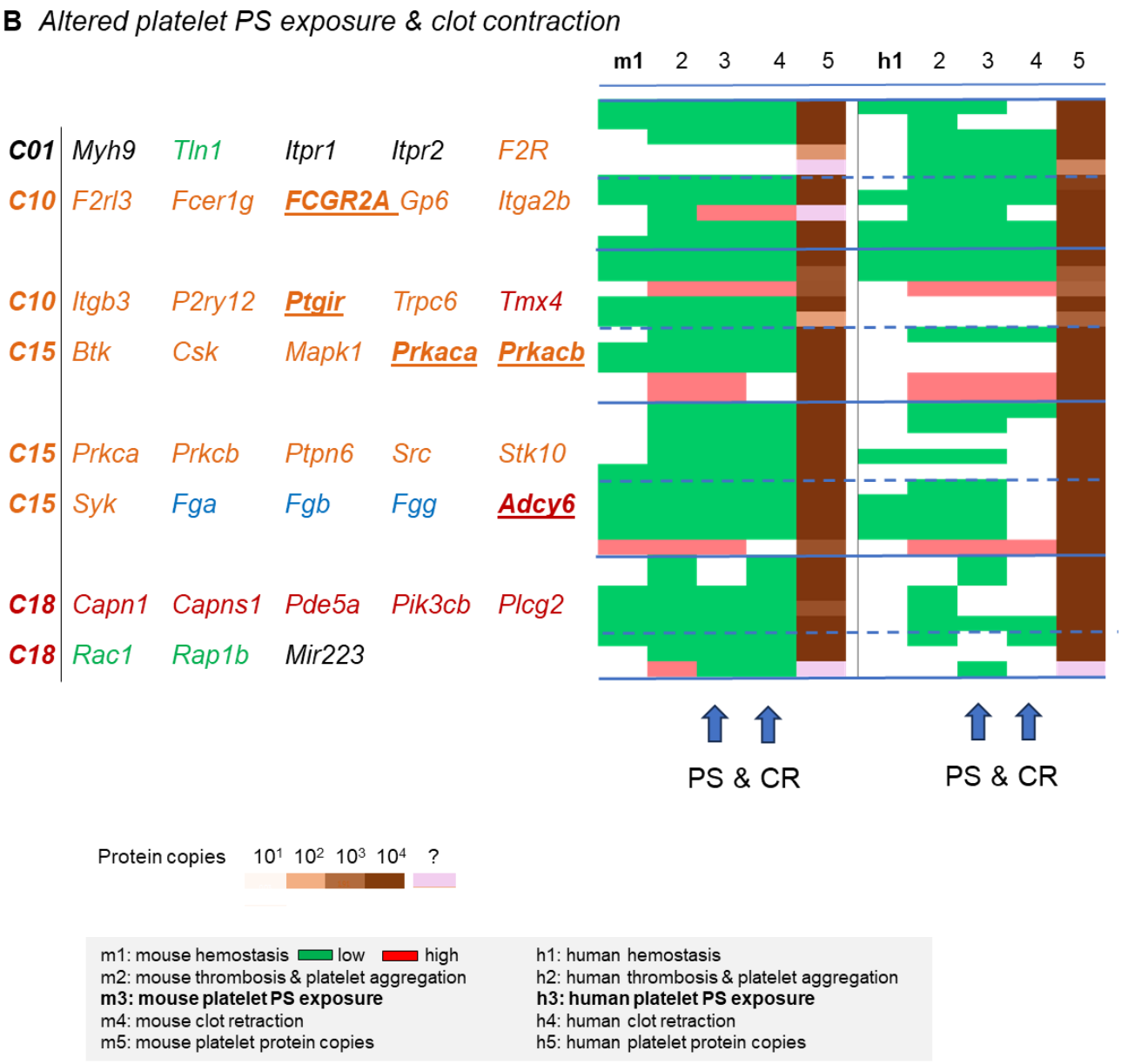
h1: human hemostasis
h2: human thrombosis & platelet aggregation
h3: human platelet PS exposure
h4: human clot retraction
h5: human platelet protein copies

A *Altered platelet PS exposure (continued)*



m1: mouse hemostasis low high
m2: mouse thrombosis & platelet aggregation
m3: mouse platelet PS exposure
m4: mouse clot retraction
m5: mouse platelet protein copies

h1: human hemostasis
h2: human thrombosis & platelet aggregation
h3: human platelet PS exposure
h4: human clot retraction
h5: human platelet protein copies



Suppl. Figure 1. Mouse genes and proteins contributing to platelet PS exposure and/or clot retraction. Earlier identified orthologous mouse and human genes with

a platelet-linked phenotype *(Huang, Blood VTH, 2025, 2:100068)* were subjected to a systematic search in PubMed ("gene/protein" & platelet & phosphatidylserine / procoagulant or clot retraction, April 2026). Listed are alphabetically, using the protein localisation and function classification scheme shown (C01-C21), all obtained mouse genes with a published phenotype of altered platelet PS exposure alone (**A**) or PS exposure plus clot retraction (**B**). Gene name colours correspond to the class colours. By default, gene deficiency resulted in lower PS exposure or clot retraction, but for the **underlined and bold** genes deficiency resulted in increases. Heatmap columns *m1-5* and *h1-5* reflect reported haemostasis, thrombosis and platelet phenotypes, due to genetic deficiency in mouse or linked to genetic variance in human.

Green colours indicate low haemostasis (bleeding), low thrombosis or platelet aggregate formation, low platelet responses, etc. **Red colours** indicate high phenotypes, **white colours** point to lack of information. Note that platelet PS exposure is scored in columns *m3/h3* and clot retraction in *m4/h4*. Protein copies are indicated by **brown colours**, as provided for mouse and human platelets *(Huang et al., Blood VTH 2025, 2:100068)*. Full data with information on gene defects or pharmacological interventions are provided in Datafile A.

Reading example**, heatmap first line nr. 2* ***Pdlim1 (class **C04**). Datafile A indicates a loss-of-function mutation of the mouse gene, phenotyped as increased arterial thrombosis and platelet aggregation, extending to increased platelet PS exposure. For the human PDLIM1 gene, no information is available. Accordingly, the heatmap contains red (high) cells for the parameters *m2* and *m3*, but no coloured cells for *h1-4*. Dark brown colours in columns *m5* and *h5* indicate high abundance in mouse and human platelets at the protein level.

Supplementary Datafile A (see Excel file). Orthologous mouse and human genes with a platelet-related phenotype were reported before (Huang, Blood VTH, 2025, 2:100068). All 4347 genes were subjected to a systematic search in PubMed ("gene/protein" & & platelet & phosphatidyl- serine/procoagulant or clot retraction, April 2026). Gene-linked alterations were included when stated as such in the original papers. **Sheet PLT PS M&H**: listed mouse/human genes with altered platelet procoagulant activity (PS exposure). **Sheet PLT PS&CR M&H:** listed mouse/human genes with altered platelet procoagulant activity (PS exposure) and altered clot retraction. Colour code: green (-1), reduced/towards bleeding; red (+1), increased, towards thrombosis.